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High glucose attenuates insulin-induced mitogen-activated protein kinase phosphatase-1 (MKP-1) expression in vascular smooth muscle cells

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Abstract

The mechanisms for the effect of hyperglycemia on insulin-induced mitogenesis were investigated using rat vascular smooth muscle cells (VSMC). VSMC were preincubated in serum-free medium with low (5 mM) glucose (LG condition) or high (25 mM) glucose (HG condition), and examined for DNA synthesis using bromodeoxyuridine (BrdUrd) incorporation. Mitogen-activated protein kinase (MAPK) activity and MAPK phosphatase (MKP-1) protein expression were detected by Western blot analysis. Phosphatidylinositol 3-kinase (PI-3K) activity was detected by thin layer chromatography. Insulin induced a dose-dependent increase in BrdUrd incorporation (123.3 ± 2.6% over basal level with 1 μM insulin) in the LG group and this effect was significantly enhanced (161.6 ± 10.4% over basal level) in the HG group. In the LG group, MAPK activity was transient with a peak activation (137.4 ± 11.2% over basal level) after 10 min exposure to 100 nM insulin. In the HG group, the MAPK activity was significantly potentiated (two-fold compared to the LG group) and was sustained even after 60 min. Insulin also induced PI-3K activity and MKP-1 expression, both of which were blocked by the PI-3K inhibitor wortmannin. In the HG group, insulin-induced PI-3K and MKP-1 expression was almost abolished. In conclusion, high glucose enhances insulin-induced mitogenesis associated with the potentiation of insulin-stimulated MAPK activity in VSMC. These effects of glucose might in part be due to the attenuation of MKP-1 expression through the blockage of the insulin–PI-3K signal pathway.

Keywords: Insulin; Glucose; Mitogen-activated protein kinase; Mitogen-activated protein kinase phosphatase-1; Phosphatidylinositol 3-kinase

1. Introduction

Insulin resistance and subsequent hyperinsulinemia are closely associated with obesity, diabetes and hypertension along with increased atherosclerotic vascular risks such as coronary artery disease [1]. The growth of vascular smooth muscle cells (VSMC) plays a central role in the genesis of atherosclerosis [2]. VSMC are responsive to insulin which experimental studies have shown to be an atherogenic hormone [3,4]; the insulin stimulates the proliferation of VSMC derived from humans and experimental animals [5,6].

Among the factors that induce insulin resistance, hyperglycemia is important in attenuating the physiological effects of insulin in such areas as glucose transport and metabolism [7,8], and a number of investigations have described these effects of hyper-

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glycemia [8–11]. Hyperglycemia induces the attenuation of insulin receptor kinase activity downstream of the receptor in several cell lines through the activation of protein kinase C and/or activation of phosphotyrosine phosphatase (PTPase) [10,12]. Recent studies suggest that hyperglycemia may also contribute to the abnormalities in VSMC through other mechanisms including the induction of insulin resistance. Elevated glucose enhances insulin-mediated rat aortic smooth muscle cell migration and proliferation [9,11] although insulin-mediated glucose transport is attenuated. Therefore, hyperglycemia associated with hyperinsulinemia could play an important role in the pathogenesis of atherosclerosis in insulin resistance. However, the mechanisms of glucose-mediated modification of the insulin signaling pathway are still unclear.

The intracellular mechanisms that mediate the mitogenic effect of insulin have been intensively investigated for a decade [13]. The mitogen-activated protein kinase (MAPK) cascade is an important signal system in mediating the mitogenic effect of growth factors including insulin [14]. Binding of insulin to its specific receptors induces a sequence of protein kinase reactions, leading to activation of MAPK kinase, MEK. MEK, the specific activator of MAPK, is a dual-specificity protein kinase that phosphorylates both threonine and tyrosine regulatory sites in MAPK. Phosphorylated and activated MAPK migrates to the nucleus, where it phosphorylates several transcription factors; the extent of protein phosphorylation is balanced by an antagonism of kinases and phosphatase. MAPK phosphatase-1 (MKP-1), a dual-specific PTPase, has been defined as an immediate early gene, which rapidly induces its mRNA and protein expression by growth factors including insulin and dephosphorylates and inactivates MAPK [15,16]. Recent studies reported that MKP-1 acts as a negative regulator of the MAPK cascade in VSMC [17–19].

We report here that high glucose attenuates insulin-induced MKP-1 expression in VSMC. Insulin-stimulated MAPK activity is enhanced and sustained in the presence of high glucose, and the mitogenic effects of insulin are also enhanced. Our findings provide new insight into the mechanisms of glucose-mediated modification of insulin action in VSMC.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL. Antibodies to p44/42 MAP kinase and its phosphospecific MAPK (Thr202, Tyr204) were obtained from New England BioLabs, and anti-MKP-1 antibody was obtained from Santa Cruz Biotechnology. Cell Proliferation ELISA kit was purchased from Boehringer Mannheim.

2.2. Cell culture

Rat aortic VSMC were isolated, as previously described [20], from medial explants of the thoracic aorta of male Sprague–Dawley rats (6–7 weeks old). VSMC were grown to confluence in DMEM containing 5 mM glucose, 10% FBS and 100 IU/ml penicillin and 100 μg/ml streptomycin. For the experiments, subculture cells from passages 4–8 at about 80% confluence in culture were used.

2.3. Bromodeoxyuridine incorporation

Cells were cultured on 96-well plates, and made quiescent by serum deprivation for 24 h in DMEM containing 0.05% bovine serum albumin instead of serum and indicated concentrations of glucose. The cells were then incubated for a further 24 h with or without insulin (1–10 000 nM). The last 4 h of this incubation period was carried out in the presence of 0.5 μM 5-bromo-2′-deoxyuridine (BrdUrd). BrdUrd incorporation was determined by ELISA, visualized, and quantified by colorimetry.

2.4. Western blot analysis

Serum-starved cells grown on 6-well plates were preincubated with the indicated concentration of glucose, then exposed to insulin (1–1000 nM) for 0–60 min. The reaction was terminated by replacement of medium with a lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EGTA, 100 μM dithiothreitol, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate and 1 mM phenylmethylsulfonyl fluoride, 5 U/ml egg white trypsin

inhibitor, 10 μg/ml leupeptin). The cells were homogenized by brief sonication, and centrifuged at 12 000 × *g* for 20 min at 4°C. The protein concentrations were determined using the Bradford protein assay [21]. Equal amounts of protein (5–10 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis was performed using anti-phosphospecific p44/42 MAPK and total p44/42 MAPK antibodies. Relative intensity of phospho-MAPK to total MAPK was quantified by densitometry.

2.5. Immunoprecipitation

Serum-starved VSMC on 100-mm dishes were exposed to insulin (100 nM) for 0–60 min. Then, the cells were washed with ice-cold phosphate-buffered saline and resuspended in lysis buffer containing 1% Triton X-100 followed by homogenization by brief sonication. The sample was centrifuged at 12 000 × *g* for 20 min at 4°C. After the protein content in resting supernatants was normalized, the sample was incubated with anti-MKP-1 antibody for 4–6 h at 4°C. Immunocomplexes were absorbed to protein A-agarose (Sigma Chemical Co.) and washed three times with lysis buffer. The immunoprecipitants were resolved by the addition of 1 × Laemmli buffer and MKP-1 protein was detected by Western blot analysis as described above. The intensity of MKP-1 protein was quantified by densitometry.

2.6. Phosphatidylinositol 3-kinase (PI-3K) assay

Immunoprecipitation was performed using anti-insulin-receptor substrate-1 (IRS-1) antibody as described above except for the detergent, 1% Nonidet p40 in lysis buffer. PI-3K activity was measured as described previously with slight modifications [19]. Briefly, the agarose-bound immunocomplexes were resuspended in 30 μl of reaction buffer (in mM: 20 Tris–HCl, pH 7.5, 10 $MgCl_2$, 1 dithiothreitol and 0.2 mg/ml phosphatidylinositol). The reaction was initiated by addition of ATP solution (25 μM [γ-^{32}P]ATP (about 10 μCi)). After incubation for 10 min at 25°C, the products were analyzed by thin layer chromatography on silica gel plates (K6, Whatman) followed by visualization of the spots by autoradiography.

2.7. Statistical analysis

Data were analyzed by Student's paired or unpaired *t*-test with a *P* value of less than 0.05 being considered significant.

3. Results

3.1. Effects of glucose on insulin-mediated DNA synthesis

Quiescent VSMC in a low glucose (5 mM) condition (LG group) were exposed to increasing concentrations of insulin, and DNA synthesis assessed by BrdUrd incorporation was measured 24 h after stimulus. Insulin stimulated BrdUrd incorporation in a dose-dependent manner. In the LG group, insulin at physiological concentration levels (from 10 nM to 1 μM) stimulated DNA synthesis (Fig. 1). Insulin-induced BrdUrd incorporation in the high glucose

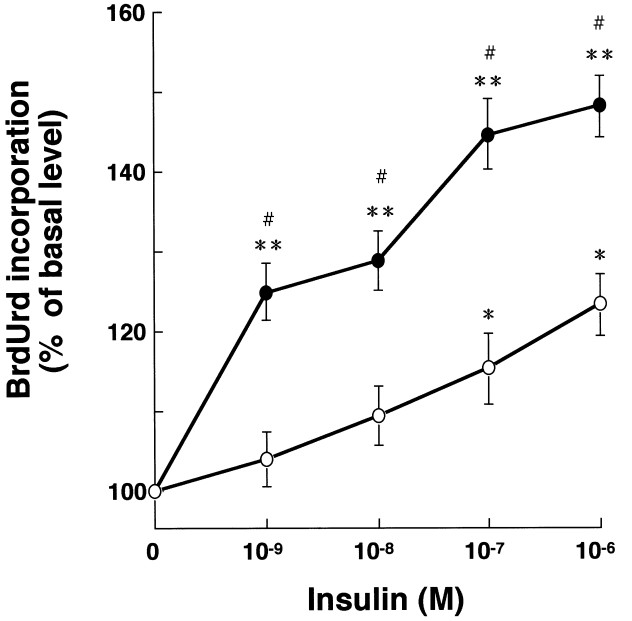

Fig. 1. Effects of glucose on insulin-mediated DNA synthesis in VSMC. Cells were serum-starved in the presence of 5 mM glucose (○) or 25 mM glucose (●), then incubated with the indicated concentrations of insulin for 24 h. BrdUrd incorporation was determined by colorimetry as described in Section 2. Results represent the mean ± S.E.M. of BrdUrd incorporation as a percent above basal level which is defined as 100% ($n = 10$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. basal value, $^{\#}P < 0.05$ vs. low glucose group).

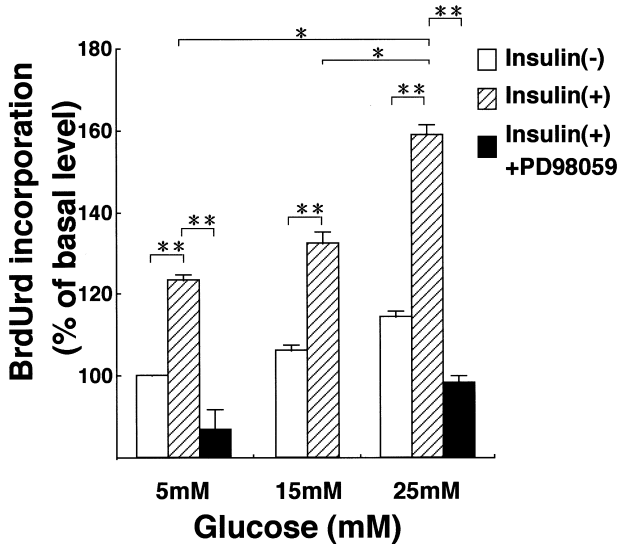

Fig. 2. Dose response of glucose on DNA synthesis in VSMC. Cells were serum-starved in the presence of the indicated concentrations of glucose for 24 h, followed by incubation with 100 nM insulin (hatched bars) or without (open bars) for 10 min. In some experiments, cells were pretreated with 50 μM PD98059 prior to exposure to insulin (closed bars). BrdUrd incorporation in VSMC was determined as described in Section 2. Results represent the mean ± S.E.M. of BrdUrd incorporation as a percent above basal level ($n=9$; $^{*}P<0.05$, $^{**}P<0.01$).

(25 mM) group (HG group) was significantly higher than that in the LG group (Fig. 1). Glucose enhanced insulin-induced DNA synthesis in a glucose concentration (from 5 to 25 mM)-dependent manner, with maximal enhancement being reached at 25 mM D-glucose (Fig. 2); thus, a glucose concentration of 25 mM was used for further experiments.

The MAPK signaling cascade is an important system in mediating the mitogenic effect of insulin [14]. To examine the role of the MAPK signaling pathway on insulin-induced DNA synthesis, we used the MEK-specific inhibitor PD98059. The cells were preincubated with low (5 mM) or high (25 mM) glucose, and treated with 50 μM PD98059 prior to exposure to insulin. PD98059 inhibited insulin-induced BrdUrd incorporation in both low and high glucose conditions (Fig. 2).

3.2. *Effects of glucose on insulin-mediated MAPK activity*

It is possible that glucose may enhance the effects of insulin on the MAPK signaling cascade corre-

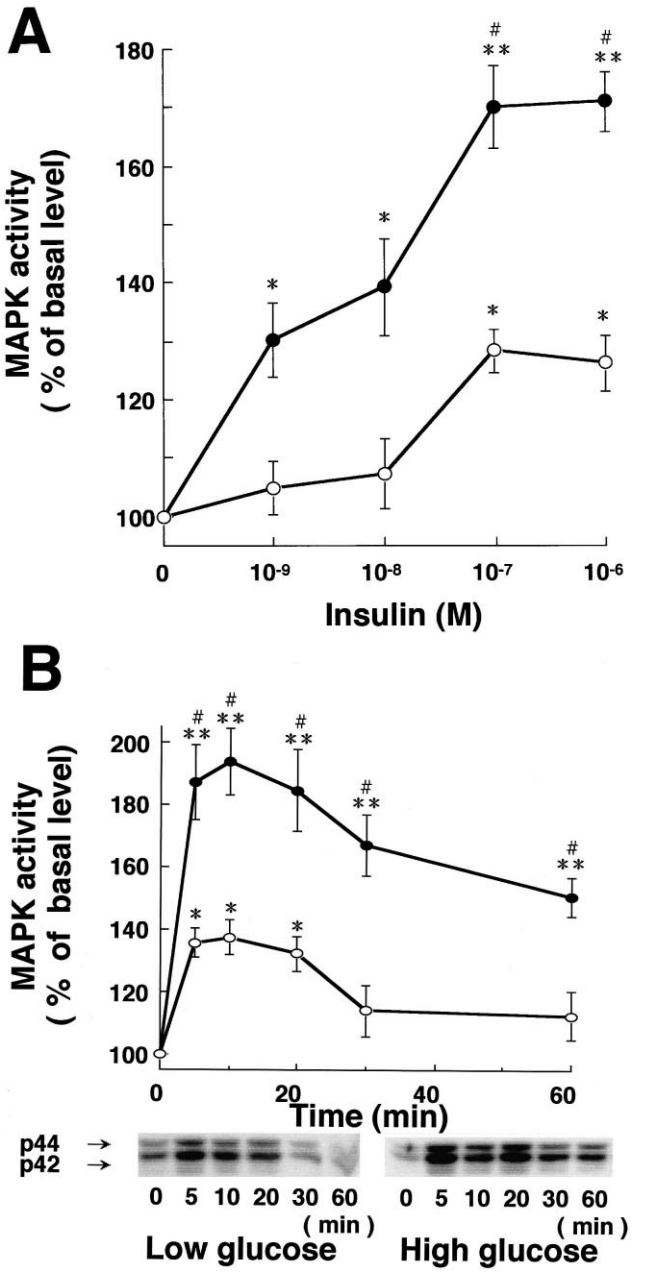

Fig. 3. Effects of glucose on insulin-induced MAPK activity in VSMC. VSMC were pretreated with 25 mM glucose (●) or without (○) for 24 h and stimulated with the indicated doses of insulin for the indicated times. Phosphorylated MAPK and total proteins were detected by Western blot analysis as described in Section 2. Data are expressed as fold MAPK activity, in which phospho-MAPK in unstimulated cells is defined as 100%. The data shown represent mean ± S.E.M. from seven separate experiments ($^{*}P<0.05$, $^{**}P<0.01$ vs. basal value, $^{\#}P<0.05$ vs. low glucose group). (A) Dose–response curve of insulin on MAPK activation in VSMC. (B) Time course of 100 nM insulin-stimulated MAPK activity. Western blot represents phospho-MAPK from single representative experiments.

sponding to the stimulated mitogenic effects in the presence of high glucose. To examine the influence of glucose on insulin-mediated MAPK activity, the cells were pretreated with 25 mM glucose (HG group) or 5 mM (LG group) for 24 h then incubated with 100 nM insulin for indicated times, and MAPK activities were estimated by Western blot analysis using anti-phosphospecific MAPK antibody. As shown in Fig. 3, in both LG and HG groups, phosphorylation of MAPK was increased in a dose- and time-dependent manner with similar ED_{50}s (about 10 nM). The peak level of MAPK activity in the HG group was significantly enhanced compared to that in the LG group (Fig. 3B). Rapid inactivation of MAPK ensued with a return to the basal level after 30 min of exposure of insulin in the LG group. In contrast, the activity was sustained up to 60 min in the HG group (Fig. 3A).

3.3. Effects of glucose on insulin-induced MKP-1 activity

Recently, it has been demonstrated that MKP-1 dephosphorylates and inactivates MAPK and plays a role in the compensatory inactivation of MAPK signaling pathways [16,17,19]. To further understand the mechanism of enhanced insulin-induced MAPK activation in the presence of high glucose, we examined the extent of MKP-1 induction in response to insulin. Quiescent VSMC, which were pretreated with 25 mM glucose (HG group) or 5 mM (LG group) for 24 h, were exposed to 100 nM insulin for the indicated times and protein extracts were prepared. Equal amounts of proteins were used to test MKP-1 protein induction using Western blot analysis. As shown in Fig. 4, in the LG group, exposure of cells to insulin resulted in a time-dependent induction of MKP-1, which was evident at 10 min and reached a maximum after 60 min incubation. In the HG group, although the basal MKP-1 level was not altered, insulin-induced MKP-1 expression was almost abolished during the 60 min incubation (Fig. 4).

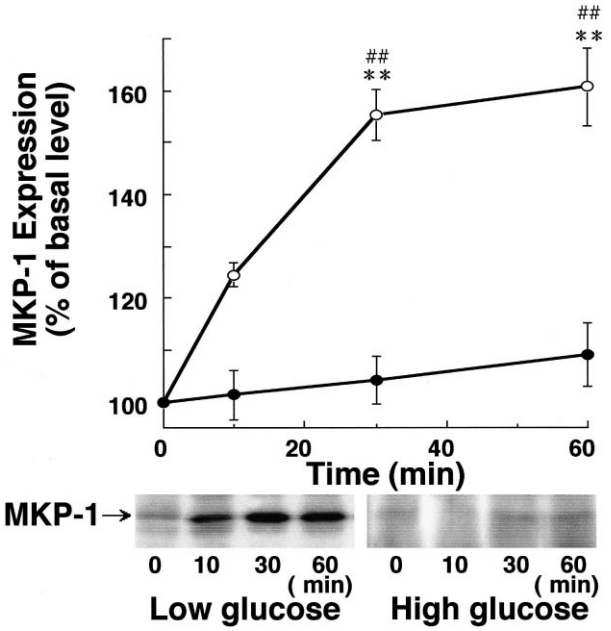

Fig. 4. Effects of glucose on insulin-induced MKP-1 expression in VSMC. VSMC were serum-starved with or without 25 mM glucose for 24 h and stimulated with 100 nM insulin for the indicated times. Expressed MKP-1 in the same amount of cell extracts was detected by Western blot analysis as described in Section 2. Data are expressed as fold MKP-1 expression, in which MKP-1 in unstimulated cells (basal level) is defined as 100%. Each value shown represents mean ± S.E.M. from nine separate experiments (**$P < 0.01$ vs. basal value, $^{\#\#}P < 0.01$ vs. low glucose group). Western blot represents MKP-1 from single representative experiments.

3.4. Effects of glucose on insulin-induced PI-3K activity in VSMC

PI-3K is an important mediator for physiological functions of insulin [13,22]. It has been reported that hyperglycemia attenuates insulin receptor kinase activity, resulting in inhibition of the activities of IRS-1 and PI-3K in several cell types [12,23]. We tested the influence of glucose on insulin-induced PI-3K activity in VSMC. As shown in Fig. 5, insulin stimulated PI-3K activity in both LG and HG conditions. However, in the HG condition, the PI-3K activity was significantly attenuated compared to that in the LG condition.

3.5. Role of PI-3K and MEK inhibitors on insulin-induced MKP-1 expression in VSMC

To examine the mechanisms of the inhibitory effect of glucose on MKP-1 induction, we tested the role of the PI-3K-specific inhibitor wortmannin on insulin-mediated MKP-1 induction. The cells were preincubated with 5 μM wortmannin for 20 min prior to exposure to insulin. As with high concentrations of

glucose, wortmannin significantly inhibited insulin-mediated MKP-1 expression (Fig. 6). Previous studies in different cell types suggest that MKP-1 induction may be regulated by MAPK [19,24]. Therefore, to evaluate the potential contribution of the MAPK signaling pathway in insulin-induced MKP-1 expression, we used the MAPK kinase inhibitor PD98059. The cells were pretreated with PD98059 for 20 min followed by insulin exposure. Insulin-induced MKP-1 expression was partially blocked by PD98059, suggesting that p44/46 MAPK contributes in part to insulin-induced MKP-1 induction (Fig. 6).

4. Discussion

We showed that high glucose weakly induced DNA synthesis and, in addition, synergistically stimulated the insulin-induced mitogenic effect in VSMC. Thus, high glucose levels might somehow affect the intracellular signaling pathway of insulin, resulting in a modification of the insulin-induced mitogenic effect. Recently, it has been demonstrated that high glucose modifies the mitogenic effects of several growth factors; under high glucose conditions, insulin stimulated 12-hydroxyeicosatetraenoic acid-induced migration of rat aortic SMC [9]. Recently, Yasunari et al. reported that high glucose treatment increases platelet-derived growth factor (PDGF)-mediated migration and even insulin-induced DNA synthesis in rabbit coronary SMC [11]. Numerous studies have investigated the molecular mechanisms of the effects of hyperglycemia. Hyperglycemia stimulates sorbitol pathway metabolism and increases the ratio of NADH/NAD^+ [25] and hyperglycemia-induced metabolic imbalances cause the production of several factors: diacylglycerol, which is known as protein kinase C activator, free radicals, and superoxide.

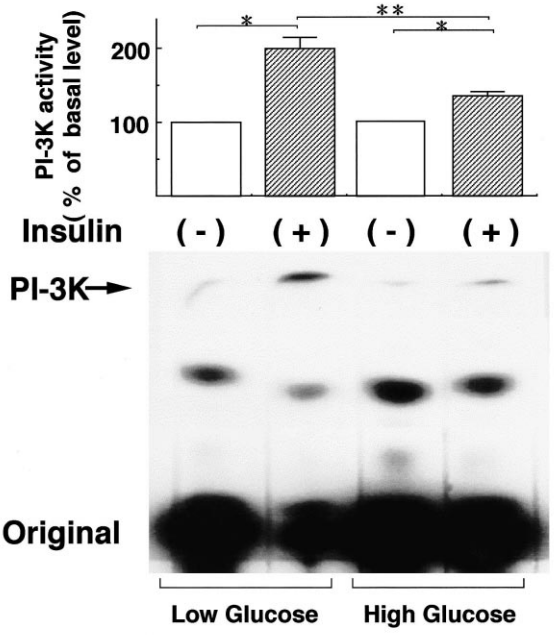

Fig. 5. Effects of glucose on insulin-induced PI-3K activity. VSMC were incubated with serum-free medium containing low (5 mM) or high glucose (25 mM) for 24 h and stimulated with 100 nM insulin (hatched bars) or without (open bars). PI-3K activities in the same amount of cell extract were detected as described in Section 2. Data are expressed as fold PI-3K activity, in which PI-3K in unstimulated cells with low glucose is defined as 100%. Each value shown represents mean ± S.E.M. from four separate experiments (*$P < 0.05$, **$P < 0.01$).

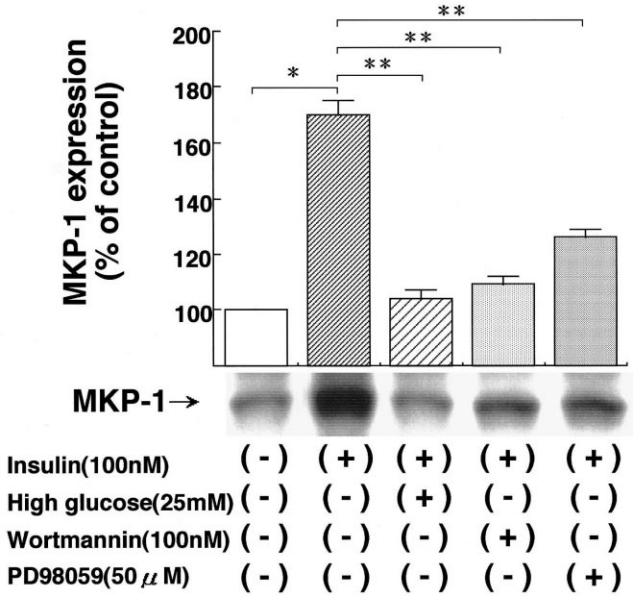

Fig. 6. Effects of wortmannin and PD98059 on insulin-induced MKP-1 expression in VSMC. VSMC were serum-starved with or without 25 mM glucose for 24 h, and pretreated with 100 nM wortmannin or 50 μM PD98059 just 20 min prior to exposure to 100 nM insulin. After 60 min incubation, expressed MKP-1 in the same amount of cell extract was detected by Western blot analysis as described in Section 2. Data are expressed as fold MKP-1 expression, in which MKP-1 in unstimulated cells (basal level) is defined as 100%. Each value shown represents mean ± S.E.M. from 10 separate experiments (**$P < 0.01$).

MAPK activation results in the activation of downstream transcriptional factors such as c-*jun* and *fos* that are required for cell proliferation [26]. MAPK has been implicated in insulin-induced growth effects [13]. In the present study, under high glucose conditions, enhanced MAPK activity is strongly associated with potentiation of insulin-induced DNA synthesis in VSMC (Figs. 1 and 3). Indeed, the MAPK kinase inhibitor PD98059 blocked insulin-induced DNA synthesis (Fig. 2). These data emphasize the importance of the mechanisms to regulate the activity of MAPK for the mitogenesis of VSMC in the presence of high glucose. The enhanced MAPK activation could have resulted from a decrease in the activity of tyrosine phosphatase and an increase in the activity of upstream participants in the ras/raf/MAPK kinase signaling cascade. Recent studies have demonstrated that MKP-1 is an important candidate phosphatase for the negative regulation on mitogen-induced MAPK activity [17,18]. In this study, insulin induced MKP-1 expression simultaneously with the activation of MAPK in VSMC, suggesting that MKP-1 plays a role in inactivation of MAPK. We also clearly showed that high glucose significantly inhibits insulin-induced MKP-1 expression. Therefore, these data suggest that sustained MAPK activity might result from the attenuation of insulin-induced MKP-1 expression under high glucose conditions.

Insulin activates the intrinsic tyrosine kinase activity of its receptor resulting in the phosphorylation of substrates such as IRS-1 [13]. These proteins act as docking molecules for other proteins via the SH2 domain, leading to the stimulation of PI-3K and the ras/raf/MAPK cascade. Recently, Begum et al. [19] reported that blockade of PI-3K signaling with its specific inhibitor wortmannin inhibited insulin-mediated nitric oxide synthase protein induction and subsequently MKP-1 expression, suggesting that insulin-induced MKP-1 expression is mediated by PI-3K-initiated signaling in VSMC. It is known that hyperglycemia induces insulin resistance, attenuating its receptor kinase activity, and subsequent decrease in IRS-1 phosphorylation [12]. We also found that insulin-stimulated PI-3K activities in VSMC are attenuated under high glucose conditions (Fig. 5). In this study, wortmannin blocked insulin-induced MKP-1 expression, an effect similar to that under high glucose conditions (Fig. 6). Together, these data suggest that insulin mediates MKP-1 expression through the PI-3K signaling pathway, and high glucose attenuates insulin-induced MKP-1 expression as a result of attenuation of PI-3K activation.

We showed in Fig. 6 that insulin-induced MKP-1 expression was partially blocked by PD98059. This is in agreement with a previous finding that p44/46 MAPK regulates in part insulin-induced MKP-1 induction [19,24]. Interestingly, we also showed that insulin-induced MKP-1 expression was attenuated whereas MAPK activity was increased in the presence of high glucose. It is not clear how to explain this discrepancy of high MAPK activity with low MKP-1 expression in high glucose conditions. However, a previous study has reported that VSMC from spontaneously hypertensive rats have a potent mitogenic response to insulin, associated with increased MAPK activity and impaired MKP-1 expression [27]. Recently, more complicated mechanisms of agonist-induced MKP-1 expression have been suggested. In VSMC, an induction of MKP-1 expression is mediated not only through the MAPK pathway but also through the stress-activated protein kinase (SAPK) and p38 MAPK pathways [24]. Moreover, arachidonic acid-induced MKP-1 expression in VSMC is mediated through tyrosine kinase [28]. The above findings suggest the existence of a complex cross-talk between the ERK/SAPK and p38 MAPK and even unidentified signaling pathways. The exact mechanisms how insulin-induced MKP-1 expression is altered in the presence of high glucose remain to be clarified.

Peak MAPK activity was reached at 10 min incubation with insulin, whereas MKP-1 was not significantly expressed (Figs. 3 and 4). In addition, basal MKP-1 protein in VSMC was not changed by high glucose. Therefore, enhanced peak MAPK activity in the high glucose condition does not account for the attenuation of MKP-1 expression. These data suggest that the enhanced peak activity of MAPK could result from the increase in the upstream MAPK cascade. Although the mechanisms by which high glucose stimulates upstream of the MAPK cascade are not yet clear, there are some candidate target proteins. Because insulin-induced IRS-1 and PI-3K are attenuated under high glucose conditions, an IRS-1-independent pathway such as the Shc/Grb/ras/raf

cascade should be present to mediate MAPK activation. The mechanisms by which high glucose stimulates upstream of the MAPK cascade have yet to be clarified.

In summary, we demonstrated that high glucose attenuated insulin-induced MKP-1 expression, which acts as a negative player in the MAPK cascade, resulting in sustained MAPK activity in VSMC. These results suggest that attenuation of MKP-1 expression may be the key mechanism for the increased VSMC growth potential of insulin under high glucose conditions.

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